



UNIVERSITI PUTRA MALAYSIA

**NEUROPROTECTIVE EFFECTS OF TOCOTRIENOL-RICH FRACTION
AND α -TOCOPHEROL OF VITAMIN E AGAINST GLUTAMATE
TOXICITY ON ASTROCYTE-NEURONAL MONO-CULTURE
AND COCULTURE SYSTEMS**

YAP HUI MIN

FPSK(m) 2013 15



**NEUROPROTECTIVE EFFECTS OF
TOCOTRIENOL-RICH FRACTION AND α -
TOCOPHEROL OF VITAMIN E AGAINST
GLUTAMATE TOXICITY ON ASTROCYTE-
NEURONAL MONO-CULTURE AND CO-
CULTURE SYSTEMS**

YAP HUI MIN

MASTER OF SCIENCE

UNIVERSITI PUTRA MALAYSIA

2013

YAP HUI MIN

MASTER OF SCIENCE

2013



**NEUROPROTECTIVE EFFECTS OF TOCOTRIENOL-RICH FRACTION
AND α -TOCOPHEROL OF VITAMIN E AGAINST GLUTAMATE
TOXICITY ON ASTROCYTE-NEURONAL MONO-CULTURE AND CO-
CULTURE SYSTEMS**

By

YAP HUI MIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Master of Science**

January 2013

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs, and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright© Universiti Putra Malaysia



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**NEUROPROTECTIVE EFFECTS OF TOCOTRIENOL-RICH FRACTION
AND α -TOCOPHEROL OF VITAMIN E AGAINST GLUTAMATE
TOXICITY ON ASTROCYTE-NEURONAL MONO-CULTURE AND CO-
CULTURE SYSTEMS**

By

YAP HUI MIN

January 2013

Chair : Huzwah Binti Khaza'ai, PhD

Faculty : Medicine and Health Sciences

Elevated concentration of glutamate, also known as glutamate neurotoxicity, is the major contributor to pathological cell death in nervous system. It has been suggested to play a key role in neurodegenerative diseases. In recent studies, palm tocotrienol-rich fraction (TRF) has been shown to exhibit better neuroprotection than α -tocopherol against glutamate toxicity. It was also shown to exert potent antioxidant, anti-inflammatory, anticancer and cholesterol-lowering properties. The main objective of this study is to elucidate the effects of TRF and α -tocopherol pre-treatment and post-treatment against glutamate toxicity in astrocyte and neuronal cell. Besides, the synergism between astrocyte and neuronal cell in protecting glutamate neurotoxicity with the supplementation of TRF and α -tocopherol were assessed through co-culture model. Astrocyte and neuronal cell in this study were exposed to high concentration of glutamate. The behavior of cell lines responding to glutamate toxicity was determined through dose-response and time course study.

Astrocyte and neuronal cell were subjected to glutamate injury before or after TRF and α -tocopherol treatment. The effects of TRF and α -tocopherol against glutamate toxicity were assessed through MTT cell viability assay, glutathione production, neuron-specific enolase (NSE) expression study and mode of cell death study. The expression of NSE was examined through reverse-transcriptase real time polymerase chain reaction (RT- qPCR) while mode of cell death was determined through acridine orange/propidium iodide (AOPI) assay. The morphological changes due to glutamate toxicity and TRF and α -tocopherol treatments were observed under fluorescence microscope. Statistical analysis was performed using one way ANOVA with SPSS 17.0. The concentration of glutamate needed to cause 50% cell death for astrocyte and neuronal cell were 230 mM and 80 mM, respectively. The concentrations of glutamate used throughout this study were only meant to cause injury to the cells. Glutamate with concentration of 60 mM and 180 mM were used to cause injury in neuronal cell and astrocyte respectively. Generally, TRF and α -tocopherol improved the cell viability of glutamate-injured neuronal cell and astrocyte by approximately 10%. In co-culture model study, TRF and α -tocopherol post-treatments provided nearly complete protection toward glutamate toxicity. Besides, TRF and α -tocopherol post-treatments were showed to restore the glutathione content upon glutamate injury. In astrocyte, TRF pre-treatment inhibited the decrease of glutathione content. In the presence of astrocyte, TRF and α -tocopherol pre-treatments inhibited decrease of glutathione content in neuronal cell which was not observed in mono-culture model. In addition, 300 ng/mL TRF and α -tocopherol completely restored glutathione production in glutamate-injured neuronal cell in co-culture model. TRF and α -tocopherol generally increased the percentage of healthy cell and decreased the percentage of necrotic cell in both cell lines as well

as in co-culture model. TRF and α -tocopherol post-treatments with concentration of 100 to 300 ng/mL decreased the percentage of necrotic cell in glutamate-injured astrocyte and neuronal cell more than 10%. Downregulation or suppression of NSE expression was observed in glutamate induced astrocyte and neuronal cell as well as in co-culture model. In conclusion, TRF and α -tocopherol provided protection and recovery properties toward astrocyte and neuronal cell against glutamate toxicity. Similar effects between TRF and α -tocopherol were found in both astrocyte and neuronal cell against glutamate toxicity. Co-culture model in this study has demonstrated synergistic properties of astrocytes and neuronal cell. Supplementation of TRF and α -tocopherol in co-culture system further improved the recovery process and protection of astrocytes and neuronal cells compared to mono-culture.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**KESAN PERLINDUNGAN SARAF OLEH FRAKSI KAYA TOKOTRIENOL
DAN ALFA-TOKOFEROL DARI VITAMIN E TERHADAP KETOKSIKAN
GLUTAMAT DALAM SISTEM MONO-KULTUR DAN KO-KULTUR SEL
NEURON-ASTROSIT**

Oleh

YAP HUI MIN

Januari 2013

Pengerusi : Huzwah Binti Khaza'ai, PhD

Fakulti : Perubatan dan Sains Kesihatan

Kepekatan glutamat berlebihan juga dikenali sebagai neurotoksisiti glutamat merupakan penyumbang utama kepada patologi kematian sel dalam sistem saraf. Ketoksikan glutamat telah dicadangkan memainkan peranan penting dalam kebanyakan penyakit degeneratif saraf. Kebelakangan ini, fraksi kaya tokotrienol (tocotrienol-rich fraction/ TRF) telah didapati lebih berkesan daripada alfa-tokoferol dalam perlindungan saraf daripada ketoksikan glutamat. TRF juga telah dibuktikan mempunyai ciri-ciri yang berkesan sebagai anti-oksidan, anti-inflamasi, anti-kanser dan merendahkan kolesterol. Tujuan utama kajian ini adalah untuk mengenalpasti kesan sebelum dan selepas penggunaan TRF dan alfa-tokoferol terhadap ketoksikan glutamate astrosit dan sel neuron. Selain itu, sinergi antara astrosit dan sel neuron dengan penggunaan TRF dan alfa-tokoferol terhadap ketoksikan glutamat juga diketengahkan dalam kajian ini dengan sistem ko-kultur. Respon sel-sel terhadap ketoksikan glutamat telah ditentukan melalui respon terhadap dos dan masa inkubasi.

Dalam kajian ini, astrosit dan sel neuron telah didedahkan kepada glutamate dalam kepekatan yang tinggi sebelum atau selepas penggunaan TRF dan alfa-tokoferol. Beberapa parameter telah dikaji bagi melihat kesan TRF dan alfa-tokoferol terhadap ketoksikan glutamat dalam astrosit dan sel neuron, termasuk ujian MTT, penghasilan glutathione, ekspresi gen 'neuron-specific enolase (NSE)' dan mod kematian sel. Ekspresi NSE dikaji melalui reaksi polymerase berantai masa nyata (real-time PCR) manakala mod kematian sel diteliti melalui mikroskop fluorescence dengan ujian 'acridine orange/propidium iodide (AOPI)'. Analisis statistik 'one way ANOVA' telah digunakan dengan SPSS 17.0. Kepekatan glutamat yang diperlukan untuk menyebabkan 50% kematian sel (IC_{50}) adalah 230 mM bagi astrosit dan 80 mM bagi sel neuron telah digunakan. Dalam kajian ini, kepekatan glutamat yang digunakan hanya bertujuan untuk mengakibatkan kecederaan kepada astrosit dan sel neuron. Glutamat pada kepekatan 180 mM bagi astrosit dan 60 mM bagi sel neuron telah digunakan. Secara keseluruhan, TRF dan alfa-tokoferol berkesan menyihatkan lebih kurang 10% sel neuron dan astrosit yang dcederakan dengan glutamat. Dalam kajian model ko-kultur, penggunaan TRF dan alfa-tokoferol selepas pendedahan glutamat memberikan perlindungan yang hampir maxima kepada ketoksikan glutamat. Selain itu, rawatan TRF dan alfa-tokoferol selepas pendedahan glutamat juga berkesan memulihkan penghasilan glutathione dalam sel neuron. Pra-rawatan TRF juga didapati berkesan menghalang pengurangan kandungan glutathione dalam astrosit. Dalam kajian model ko-kultur, 300 ng/mL TRF dan alfa-tokoferol memulihkan penghasilan glutathione sepenuhnya dalam sel neuron yang telah dcederakan oleh glutamat. TRF dan alfa-tokoferol secara keseluruhannya meningkatkan peratusan sel sihat dan mengurangkan peratusan sel nekrotik dalam sel neuron, astrosit dan juga ko-kultur. Rawatan TRF dan alfa-tokoferol dengan

kepekatan 100 kepada 300 ng/mL selepas pendedahan glutamat dalam astrosit dan sel neuron menurunkan peratus sel nekrotik lebih daripada 10%. Diamati bahawa ekspresi NSE diturunkan atau dihalang dalam astrosit dan sel neuron yang dcederakan oleh glutamat. Kesimpulannya, TRF dan alfa-tokoferol memberikan perlindungan dan pemulihan bagi astrosit dan sel neuron daripada ketoksikan glutamat. Didapati kesan yang sama antara TRF dan alfa-tokoferol dalam sel astrosit dan neuron menentang ketoksikan glutamat. Model ko-kultur dalam kajian ini telah menunjukkan ciri-ciri sinergi antara sel astrosit dan neuron. Penggunaan TRF dan alfa-tokoferol dalam sistem ko-kultur meningkatkan lagi proses pemulihan dan perlindungan astrosit dan sel neuron daripada ketoksikan glutamat jika dibandingkan dengan sistem mono-kultur.

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my supervisor, Dr. Huzwah binti Khaza'ai, for the help, guidance, encouragement and advice throughout my project. I would like to thank Assoc. Prof. Dr. Sokhini bin Abd Mutalib and Dr. Abdah binti Akim for their helps and advices. Besides, I would like to thank Prof. Daud, Assoc. Prof. Dr. Cheah Yoke Kqueen, and Assoc. Prof. Dr. Chong Pei Pei for the use of the lab facilities.

I would like to extend my gratitude to all the lab officers in Cell Signaling and Biochemistry Labs for assisting me in carrying out the experiment, especially Mr. Zul. I would also like to thank my seniors, Mr. Ibrahim and Miss Ainun, for their guidance and encouragement. I also would like to express my gratitude to my family for their timeless support. Last but not least, I would like to take this opportunity to thank my friends, especially Lye Kwan Liang, Tung En En, Hazirah, Nurhayatie, Thilaga, Lee Pey Yee and Alan Tan, for helping and sharing experiences throughout my study.

I certify that a Thesis Examination Committee has met on 15 January 2013 to conduct the final examination of Yap Hui Min on her thesis entitled “Neuroprotective Effects of Tocotrienol-rich Fraction and α -tocopherol of Vitamin E against Glutamate Toxicity on Astrocyte-neuronal Mono-culture and Co-culture Systems” in accordance with the Universiti and Universiti College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Mohd Nasir bin Mohd Desa, Phd

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Roslida binti Abd Hamid @ Abdul Razak, Phd

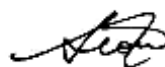
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Asmah binti Rahmat, Phd

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Durriyyah Sharifah Hasan Adli, Phd

Associate Professor
Institute of Biological Sciences
Universiti Malaya
(External Examiner)



Seow Heng Fong, Phd
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 March 2013

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Huzwah Binti Khaza'ai, PhD

Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Abdah Binti MD Akim, PhD

Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)



BUJANG BIN KIM HUAT, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

YAP HUI MIN

Date: 1 August 2013

TABLE OF CONTENTS

	ABSTRACT	Page
	ABSTRAK	ii
	ACKNOWLEDGEMENTS	v
	APPROVAL	viii
	DECLARATION	ix
	LIST OF TABLES	xi
	LIST OF FIGURES	xv
	LIST OF APPENDICES	xvii
	LIST OF ABBREVIATIONS	xxi
	LIST OF ABBREVIATIONS	xxiii
 	CHAPTER	
1	INTRODUCTION	1
	1.1 Background of study	1
	1.2 Problem statement	4
	1.3 Research objectives	4
	1.3.1 General objective	4
	1.3.2 Specific objectives	4
	1.4 Hypotheses	5
 	2	6
	LITERATURE REVIEW	
	2.1 Nervous system	6
	2.1.1 Neuronal cell	7
	2.1.2 Glial cell	8
	2.1.3 Neuron-astrocyte interaction in the brain	8
	2.2 Neurodegenerative diseases	9
	2.2.1 Alzheimer's disease (AD)	10
	2.2.2 Parkinson's disease (PD)	11
	2.2.3 Other neurodegenerative diseases	12
	2.3 Factors involved in neurodegenerative diseases	13
	2.3.1 Astrocytes in neurodegenerative diseases	13
	2.3.2 Glutamate	14
	2.3.3 Oxidative stress	23
	2.4 Mode of cell death	25
	2.5 Antioxidant	27
	2.6 Glutathione (GSH)	27
	2.7 Vitamin E	29
	2.7.1 Metabolism of vitamin E	31
	2.7.2 Isomers of vitamin E	33
	2.7.3 Functions of vitamin E	36
	2.8 Biomarker of neuronal cell injury	38
 	3	40
	MATERIALS AND METHODS	
	3.1 Cell culture	40
	3.1.1 Cell thawing	41
	3.1.2 Cell storage	41
	3.1.3 Subculturing of cell	42
	3.1.4 Cell seeding	43

3.2	Cell viability assay	43
3.2.1	Dose response studies	44
3.2.2	Time course	45
3.2.3	Viability of glutamate-injured cell upon TRF and α -tocopherol treatments	46
3.6	Glutathione assay	51
3.6.1	Glutathione production of glutamate-injured cell upon TRF and α -tocopherol treatments	51
3.6.2	Glutathione colorimetric assay	52
3.7	Mode of cell death studies	53
3.7.1	Morphological study of glutamate-injured cell upon TRF and α -tocopherol treatments	53
3.7.2	Acridine orange/propidium iodide (AO/PI) assay	54
3.8	Neuron-specific enolase expression studies	55
3.8.1	Expression of neuron-specific enolase (NSE) in glutamate-injured cell upon TRF and α -tocopherol treatments	55
3.8.2	RNA extraction	56
3.8.3	RNA concentration and RNA purity determination	57
3.8.4	Reverse transcription and cDNA synthesis	58
3.8.5	Polymerase chain reaction (PCR)	58
3.8.6	Gel electrophoresis	60
3.8.7	Real time PCR	61
3.8.8	Purification and sequencing of PCR product	62
3.9	Statistical analysis	63
4	RESULTS	64
4.1	Glutamate dose response studies	64
4.2	Time course studies for glutamate	66
4.3	Viability of glutamate-injured cell upon TRF and α -tocopherol treatments	67
4.3.1	Neuronal cell	68
4.3.2	Astrocyte	72
4.3.3	Astrocyte-neuronal co-culture	76
4.4	Glutathione production of glutamate-injured cell upon TRF and α -tocopherol treatments	78
4.4.1	Neuronal cell	79
4.4.2	Astrocyte	81
4.4.3	Astrocyte-neuronal co-culture	83
4.5	Morphological study of glutamate-injured cell upon TRF and α -tocopherol treatments	85
4.5.1	Neuronal cell	86
4.5.2	Astrocyte	92
4.5.3	Astrocyte-neuronal co-culture	97
4.6	Expression of neuron-specific enolase (NSE) of glutamate-injured cell upon TRF and α -tocopherol treatments	102
4.6.1	Neuronal cell	103
4.6.2	Astrocyte	105
4.6.3	Astrocyte-neuronal co-culture	107

5	DISCUSSION	109
5.1	Dose response and time course study	109
5.2	Viability of glutamate-injured cells upon TRF and α -tocopherol treatments	111
5.3	Glutathione production of glutamate-injured cells upon TRF and α -tocopherol treatments	114
5.4	Mode of cell death of glutamate-injured cells upon TRF and α -tocopherol treatments	117
5.5	Expression of neuron-specific enolase (NSE) of glutamate-injured cells upon TRF and α -tocopherol treatments	119
6	CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH	122
	REFERENCES	126
	APPENDICES	148
	BIODATA OF STUDENT	183
	PUBLICATIONS	184